

Micropropagation of loblolly pine by somatic organogenesis and RAPD analysis of regenerated plantlets

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Abstract Organogenesis was induced in callus derived from mature zygotic embryos of six families (J-56, S-1003, E-22, E-311, E-440, and Mc) of loblolly pine (*Pinus taeda* L.) within 24 weeks of culture. Elongation of adventitious buds was achieved on TE medium supplemented with 0.5 mg · L⁻¹ indole-3-butyric acid (IBA) and 1 mg/l 6-benzyladenine (BA). The most suitable medium for root formation proved to be TE medium supplemented with 0.5 mg · L⁻¹ IBA, 2 mg · L⁻¹ BA, and 0.5 mg/l gibberellic acid (GA3). 169 regenerated plantlets were transferred to a perlite : peatmoss : vermiculite (1:1:1) soil mixture, and 98 plantlets survived in the field. Total DNA was extracted from the needles of the regenerated plantlets of the six families of loblolly pine. Analysis of random amplified polymorphic DNA (RAPD) using 20 arbitrary oligonucleotide 10-mers, show that amplification products were monomorphic for all the plantlets of family J-56, S-1003, E-22, E-311, E-440, and Mc of loblolly pine. These results suggested that organogenesis can be used for clonal micropropagation of some families of loblolly pine.

Key words: *Pinus taeda* L., Organogenesis, Plant regeneration, Random amplified polymorphic DNA (RAPD)

Introduction

Organogenesis and somatic embryogenesis have been regarded as the in vitro system of choice with the potential for eventual mass propagation of superior and genetically engineering forest tree genotypes in both coniferous and hardwood (Gupta *et al.* 1991; Becwar *et al.* 1995). Somatic embryogenesis and organogenesis have been induced from more than 30 tree species in conifers, but plant regeneration via somatic embryogenesis and organogenesis remains difficult with a low regeneration frequency (Attree *et al.* 1993; Kaul *et al.* 1985; Laliberter *et al.* 1988; Gladfelter *et al.* 1987). Loblolly pine (*Pinus taeda* L.) is an economically important forest tree, widely planted in tropical and subtropical regions (Becwar *et al.* 1995). Litvay *et al.* (1981) and Teasdale *et al.* (1986) obtained calli and suspension cultures from loblolly pine (without describing plantlet regeneration). In vitro propagation of this coniferous species via somatic embryogenesis and organogenesis from callus induced from immature zygotic embryos and cotyledons has been reported (Mott *et al.* 1981; Gupta *et al.* 1987; Sen *et al.* 1989; Becwar *et al.* 1990; Jang *et al.*

1991; Handley *et al.* 1995). Plant regeneration via organogenesis from callus derived from mature zygotic embryos has been achieved (Tang *et al.* 1998). However, work to improve regeneration frequency and establish a regeneration protocol suitable for most families of loblolly pine is still needed.

Molecular markers, such as restriction fragment length polymorphism (RFLP) (Atchison *et al.* 1976; Vedel *et al.* 1976), random-amplified polymorphic DNA (RAPD) (Williams *et al.* 1990), and amplified fragment length polymorphism (AFLP) (Vos *et al.* 1995) appear to be powerful tools in the identification of plant species. They had been used for confirming somatic hybrids (Takemori *et al.* 1994), determining the geographic variation of medical plants (Nakai *et al.* 1996), evaluating the genetic relationship between the plant species and their secondary metabolites (Yamazaki *et al.* 1994), and conducting the genetic analysis of micropropagated plantlets (Rani *et al.* 1995; Shoyama *et al.* 1997). There are no reports on RAPD analysis of regenerated plantlets from organogenesis in coniferous species. In our study of the application of plant tissue culture to the clonal propagation of coniferous species, we decide to use the technique of RAPD analysis to identify the regenerated plantlets and to confirm the probably genetic homogeneity and genetic variation of regenerated plantlets in some families of loblolly pine. In the present investigation, we report the in vitro regeneration

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of six families of loblolly pine and the RAPD analysis of regenerated plantlets from callus derived from mature zygotic embryos on the basis of a previously described procedure that involved plant regeneration via organogenesis (Tang *et al.* 1998).

Materials and methods

Plant materials

Mature seeds of six families of loblolly pine were collected from 25 to 30-year old trees in October 1996, from Yingde Seed Orchard (E-22, E-311, E-440, and Mc) at Yingde, Guangdong Province, China, and the southeast United States (J-56, S-1003). All seeds were stored in plastic bags at 4°C for at least two months before they were used to tissue culture. Seeds were disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 0.1% mercuric chloride for 20 min, followed by four to five rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the megametophytes and placed horizontally on a solidified callus induction medium in flasks. Each treatment consisted of 150-200 explants, and the experiment was repeated three to five times.

Callus and adventitious bud induction

Mature zygotic embryo explants were cultured on callus induction medium consisted of TE medium supplemented with 10 mg/L (-naphthaleneacetic acid (NAA), 4 mg · L⁻¹ benzyladenine (BA), and 2 mg · L⁻¹ kinetin. The induction frequency of callus from mature zygotic embryos was determined after 8 weeks on callus induction medium. Callus were subcultured on the callus proliferation medium with auxin and cytokinin concentration lowered to 1/5. After 6 weeks, light-yellowish, loose, glossy globular callus were selected for differentiation. The differentiation medium consisted of DCR (Gupta *et al.* 1985), LP (von Arnold and Eriksson 1976), MSG (Becwar *et al.* 1990), MS (Murashige and Skoog 1962), SH (Schenck and Hildebrandt 1972), and TE (Tang *et al.* 1998), containing 0.5 mg · L⁻¹ indole-3-butyric acid (IBA), and 2 mg · L⁻¹ BA, respectively. The differentiation frequency of adventitious buds and mean number of adventitious buds per gram callus were counted after 12 weeks on differentiation medium. Each treatment was replicated four times, and each replicate consisted of 90 callus tissues, averaging 1.2 gram per tissue.

Shoot rooting and plant regeneration

Adventitious buds more than 1 cm in height were selected for rooting. Rooting medium consisted of TE medium containing 0.1 mg · L⁻¹ IBA, 1 mg · L⁻¹ BA, and 0.5 mg · L⁻¹ gibberellic acid (GA3) and 1000 mg · L⁻¹ activated charcoal. Each treatment was replicated

three to five times, and each replicate consisted of 30 explants. Rooting frequency was counted in the 6th week of culture. Plantlets obtained from shoots rooted on agar medium were taken out of the culture flasks, washed under running tap water to remove agar, transferred to plastic cups containing sterilized soilrite, and kept in a culture room at 24 ± 1 °C and 16 h/day illumination with cool fluorescent light. The plantlets were irrigated every day with 2-3 mL tap water. After 4-5 weeks, plantlets were transferred into the field. In callus induction, adventitious bud differentiation, and rooting medium, the concentrations of sucrose, casein hydrolysate, glutamine, and agar were 30 g · L⁻¹, 400 mg · L⁻¹, 400 mg · L⁻¹, and 7 g · L⁻¹, respectively. The pH was adjusted to 5.8 with 1N KOH or 0.5 N HCl prior to autoclaving at 121 °C for 18 min. Callus induction took place in the darkness at 21°C and adventitious bud differentiation, elongation, and rooting took place in the light with 12 h photoperiod at 23 °C. Data were analyzed with analysis of variance, and mean comparison was made with the least significant difference test at 5% level of probability.

DNA isolation and RAPD analysis

DNA was isolated from mature zygotic embryos and regenerated plantlets of six families of loblolly pine by a slight modification of the method of the Devey *et al.* (1991). Needles tissue were cut into small pieces, ground in liquid nitrogen, and homogenized with a Brinkmann polytron at 4 °C in 15 mL of extraction buffer [50 mM Tris (pH8.0), 5 mM EDTA, 0.35 M sorbitol, 0.1% BSA, 0.1% mercapto ethanol, 10% wt/vol polyvinyl pyrrolidone (M. wt 40 000)]. The homogenate was centrifuged at 3 000 rpm, at 4°C for 10 min. The pellet was resuspended in 1.5 mL of buffer [50 mM Tris (pH8.0), 25 mM EDTA, 0.35 M sorbitol, 0.1% mercapto ethanol]. The suspension was brought to a final concentration of 1% (w/v) N-laurylsarcosine, 0.7M sodium chloride, and then to 1% (w/v) with hexadecyltrimethylammonium bromide (CTAB) and incubated at 60°C for 30 min. The same was centrifuged at high speed and the aqueous layer precipitated with 2/3 vol isopropanol, 0.03M ammonium acetate. The DNA was dissolved in 50 (l of tris EDTA, pH 8.0. The concentration of DNA was read on a fluorometer. Amplification was performed in a 50 (l reaction mixture containing 200 (M dNTPs, 0.25 (M primer, 2U Taq DNA polymerase (Promega), 1.5 mM MgCl₂, 100 ng genomic DNA, and 5 (l 10 (Taq polymerase buffer in a programmable AmpliTaq Thermalcycler for 30 cycles of 40 s at 94 °C, 1 min at 50 °C, and 1.5 min at 72 °C. The amplification products were resolved by electrophoresis in 0.8% agarose gel, stained with ethidium bromide, and photographed by a DNA transilluminator. The size of

the amplification products was estimated for a 1 kb ladder (Pharmacia). Random primers, each consists of 10 nucleotides (OPB and OPO series), were purchased from Operon Technologies.

Scanning electron microscopy

Organogenic callus were prepared for scanning electron microscopy according to Attree *et al.* (1993) with some modification. Tissues were fixed overnight in 4% glutaraldehyde and 100 mM phosphate buffer (pH7.0), wash one time in 100 mM phosphate buffer (pH7.0) for 30 min, followed by dehydration in successive ethyl alcohol solution of 85, 95, and 100%, each repeated twice for 5 min. Specimens were dried in a critical-point-drier with CO₂ for 2 h, mounted on Cu stubs and gold-coated. The samples were examined and photographed in HITACHI scanning electron microscope.

Results and discussion

Callus induction

Investigation on the plantlet regeneration through somatic embryogenesis in loblolly pine had been carried out by some workers (Gupta *et al.* 1991; Becwar *et al.* 1995). Somatic embryogenesis of loblolly pine has usually been induced from immature zygotic embryos, which requires a long period, with a low embryogenic callus induction frequency. Our previous investigation indicated that mature zygotic embryo culture was the most effective cultured method in inducing plantlet regeneration via organogenesis in loblolly pine as compared with the culture of other organs. (Tang *et al.* 1998). Therefore, mature zygotic embryos were cultured on the callus induction medium consisted of TE medium supplemented with 10 mg/L NAA, 4mg · L⁻¹ BA, and 2 mg · L⁻¹ kinetin in the dark. All mature zygotic embryos produced callus within 12 weeks. As the calli started to proliferate, three types could be distinguished: light-yellowish, loose, glossy globular (Fig. 3a), light-green globular, and light-brown globular. Light-yellowish, loose, glossy globular callus was initiated from 36.2%, 58.3%, 13.7%, 14.9%, 16.8%, and 29.7% of the J-56, S-1003, E-22, E-311, E-440, and Mc mature zygotic embryos, respectively, on TE callus induction medium supplemented with NAA, BA, and kinetin (Fig. 1). Light-yellowish, loose, glossy globular callus developed from the cotyledons of the mature zygotic embryos. Both light green globular and light brown globular calli formed from the hypocotyl as well as from the radicle of the explants. Light-yellowish, loose, glossy globular callus was morphogenic, and was selectively proliferated on TE callus proliferation medium with auxin and cytokinin concentration decreased

to 1/5.

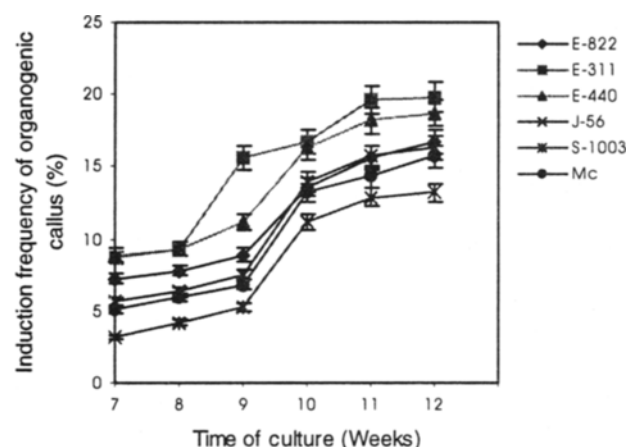


Fig 1. The influences of families of loblolly pine on morphogenic callus induction frequency (%)

Each treatment was replicate four times, and each replicate consisted of 200 mature zygotic embryo explants. Values represent the means \pm S.D.

Differentiation of adventitious buds

Nine weeks after proliferation culture, light-yellowish, loose, glossy globular callus was transferred to differentiation media consisted of DCR, LP, MS, MSG, SH, and TE, respectively, supplemented with 2 mg · L⁻¹ BA and 0.5 mg · L⁻¹ IBA. The differentiation frequency of adventitious buds was counted in the 12th week of culture. The results showed that the highest differentiation frequencies of 31.2%, 57.6%, 16.9%, 21.9%, 16.3%, and 29.1% for J-56, S-1003, E-22, E-311, E-440, and Mc, respectively, were obtained on TE medium containing 0.5 mg/L IBA and 2 mg/L BA (Table 1). In a culture period of 12 weeks, calli derived from mature zygotic embryos of family J-56, S-1003, E-22, E-311, E-440, and Mc, incubated in TE medium with 0.5 mg · L⁻¹ IBA and 2 mg/L BA, had the highest mean number of adventitious buds per g of callus, 4.3, 5.7, 2.1, 1.5, 1.8, and 2.3, respectively (data not shown). Adventitious buds formed at much lower frequencies, when DCR, LP, MS, MSG, SH were used as basic medium for differentiating adventitious buds. Adventitious bud primordia were formed from callus cultures in the third week of differentiation culture (Fig. 3b-c). Adventitious buds of 0.1-0.5 cm in height were developed from calli by the 6th week (Fig. 3d). The development of adventitious buds was asynchronous. There were often many small adventitious buds and bud primordia surrounding the larger shoots. Several adventitious buds were formed on the same callus. After adventitious buds of 0.3-0.5 cm in height were transferred to fresh differentiation medium, a second cluster of adventitious buds often developed from the callus.

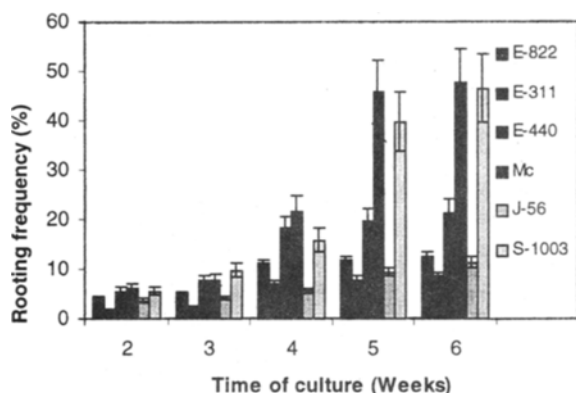
Table 1. The influences of basic media on adventitious bud differentiation frequency (%) of six families of loblolly pine

Basic media	Families of loblolly pine (%response)					
	J-56	S-1003	E-22	E-311	E-440	Mc
DCR	21.1±1.7b	23.2±2.1b	14.2±1.2b	12.7±1.2b	11.7±1.0b	16.5±1.4b
LP	18.7±1.3b	15.6±1.2b	13.1±1.3b	14.3±1.3b	13.6±1.2b	15.2±1.3b
MS	3.2±1.0c	3.8±0.8c	2.1±0.8c	2.3±1.0c	1.8±0.8c	2.8±0.5c
MSG	5.7±0.8c	5.9±1.0c	3.5±0.8c	5.7±0.8c	4.7±0.8c	4.7±1.0c
SH	7.5±1.0c	6.7±1.0c	5.2±1.2c	7.2±1.0c	6.5±1.0c	5.9±1.2c
TE	31.2±1.4a	57.6±2.3a	16.9±1.2a	21.9±1.8a	16.3±0.8a	29.1±2.1a

Note: Each treatment was replicate four times, and each replicate consisted of 90 callus tissues, averaging 1.2 g per tissues. Values represent the means ± S.D. Means followed by the same letter are not significantly different at the 0.05 level of confidence.

Shoot rooting and plant regeneration

Transferring developing adventitious buds to fresh differentiation media resulted in stem elongation and growth of whole adventitious buds. The *in vitro* rooting of adventitious shoots was achieved in 10.3%~46.7% for six families of loblolly pine (J-56 10.3%, S-1003 46%, E-22 12.6%, E-311 8.7%, E-440 21.3%, and Mc 46.7%) after adventitious shoots were cultured on rooting medium consisted of TE medium supplemented with 0.1 mg · L⁻¹ IBA, 1 mg · L⁻¹ BA, and 0.5 mg · L⁻¹ GA3 for 6 weeks (Fig. 2).

**Fig. 2.** The influences of families of loblolly pine on rooting frequency (%)

Each treatment was replicate four times, and each replicate consisted of 30 adventitious shoots. Values represent the means (S.D.)

Adventitious shoots cultured on medium without GA3 failed to root. Most commonly, a single root developed from each adventitious shoot. In some cases secondary root development was also observed. After hardening-off for 7-10 d, a total of 169 rooted adventitious shoots derived from six families sources of loblolly pine were successfully transferred to soil during 1997 and 1998, with 98 plantlets surviving in the soil. Compared with the plantlet regeneration system reported in conifers (Attree *et al.* 1993), our results show that we have achieved a relatively high-

frequency *in vitro* propagation system which is probably suitable for most of families of loblolly pine. On the basis of this regeneration system, we can conduct research on gene transfer into loblolly pine, and propagate the superior families from traditional breeding method of loblolly pine. In order to confirm the probably genetic homogeneity, we have used the technique of RAPD analysis to identify the regenerated plantlets from six families of loblolly pine.

RAPD analysis of regenerated plantlets

RAPD markers are useful tools in genetic identification of micropropagation plants (Rani *et al.* 1995; Shoyama *et al.* 1997). In order to confirm whether or not the *Pinus taeda* plantlets regenerated through organogenesis from callus derived from mature zygotic embryos might be genetically homogeneous, we have compared the RAPD patterns of regenerated plantlets from family J-56, S-1003, E-22, E-311, E-440, and Mc of loblolly pine with that of the original plants. RAPD analysis using 20 random primers of the total DNA extracted from the needles of plantlets regenerated from callus and from that of the original plant gave the amplified band pattern. Fig. 3e show representative amplified band pattern produced by the primers OPO-08 in regenerated plantlets from family S-1003 of loblolly pine. Fig. 3f shows a representative amplified band pattern produced by primer OPB-05 in regenerated plantlets from family E-311 of loblolly pine. All of primers tested produced representative amplified band pattern that monomorphic in regenerated plantlets tested from family J-56, S-1003, E-22, E-311, E-440, and Mc of loblolly pine. A single leaf segment of the regenerated plantlets was sufficient for RAPD analysis. Therefore, this assay system may prove to be useful for determining both genetic homogeneity and somatic variation of loblolly pine plantlets regenerated by organogenesis. RAPD fingerprinting of the total DNA extracted from the leaves of loblolly pine regenerated from J-56, S-1003, E-22, E-311, E-440, and Mc family of loblolly pine and from that of the parental plant gave the same patterns. These results suggest that organogenesis from callus

derived from mature zygotic embryos of family J-56, S-1003, E-22, E-311, E-440, and Mc of loblolly pine

may be used routinely for clonal micropropagation.

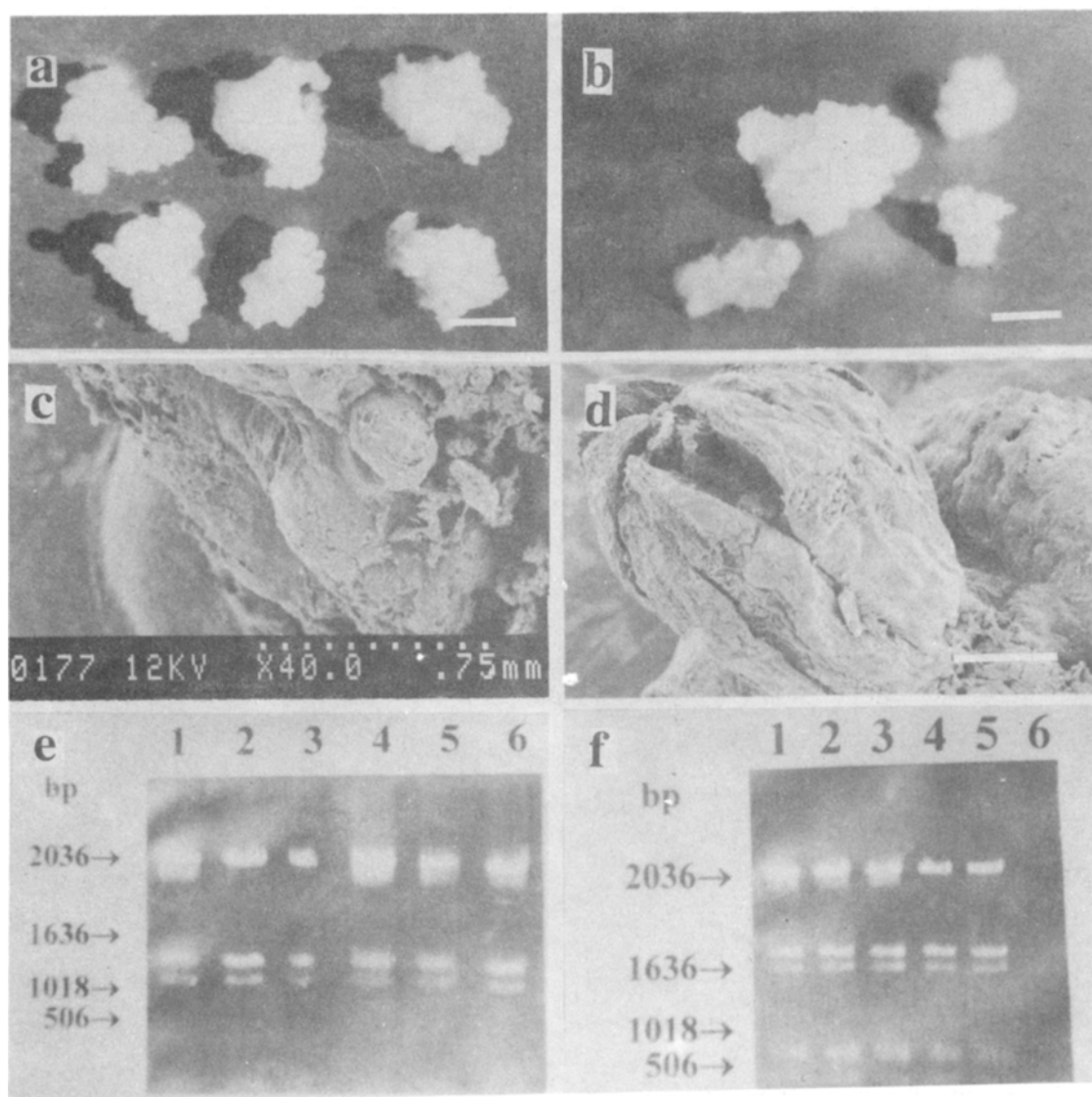


Fig. 3 In vitro regeneration of loblolly pine and RAPD analysis of regenerated plantlets.

(a) Light-yellowish, loose, glossy globular callus (bar = 0.5 cm), (b) differentiation of organogenic calluses (bar = 0.5 cm), (c) preliminary adventitious buds, (d) adventitious shoot (bar = 0.5 mm), (e) polymorphic bands in family S-1003 of loblolly pine as revealed by gel electrophoresis of RAPD fragment generated with primers OPO-08 (5'-CCTCCAGTGT-3') (lane 1 mature zygotic embryos of family S-1003 of loblolly pine, lane 2-6 regenerated plantlets, left molecular markers indicated in bases), (f) polymorphic bands in family E-311 of loblolly pine using primers OPB-05 (5'-TGCGCCCTTC-3') (lane 1 mature zygotic embryos of family E-311 of loblolly pine, lane 2-5 regenerated plantlets, lane 6 control with water, left molecular markers are indicated in bases)

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